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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **334th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, St Bartholomew's Medical College, Charterhouse Square, London, E.C. 1 on Friday, 12 November 1954, at 2 p.m., when the following papers were read:*

COMMUNICATIONS

The Effect of Chlorate on the Rate of Nitrite Oxidation by *Nitrobacter*. By H. Lees and J. R. Simpson. (*Department of Biological Chemistry, University of Aberdeen*)

Nitrobacter is an autotrophic micro-organism which obtains its entire supply of energy by oxidizing nitrite to nitrate; it is generally assumed to be responsible for nitrite oxidation in soil. Chlorate (10^{-4}–10^{-5} M) apparently inhibits the growth of the organism in soil without affecting its ability to oxidize nitrite (Lees & Quastel, 1945). At higher concentrations (10^{-3} M) chlorate also inhibits nitrite oxidation (Quastel & Scholefield, 1951).

We have now studied the action of chlorate on resting suspensions of *Nitrobacter* by the Warburg technique. Enrichment cultures of *Nitrobacter* were grown in strongly aerated suspensions of calcium phosphate. The bacteria adhered to the calcium phosphate particles, from which they were removed by a brief treatment in an 'Atomix' homogenizer. They were finally separated from the calcium phosphate, and concentrated, by differential centrifuging.

The concentrated suspensions of *Nitrobacter* so prepared oxidized nitrite at a linear rate (O_2 uptake 0·1–0·2 μmole/min.) in the absence of chlorate. When chlorate (2×10^{-2}–2×10^{-3} M) was added, the rate of nitrite oxidation progressively decreased from the time of addition. Cells pre-incubated with chlorate and nitrite and then washed showed a diminished rate of nitrite oxidation. Cells pre-incubated with chlorate alone, and then washed, showed a normal rate of oxidation.

These and other results agree with the theory that chlorate displaces nitrate from, and combines irreversibly with, a nitrite-oxidizing enzyme (E) at the stage during nitrite oxidation at which the enzyme is carrying a nitrate ion, e.g.

$$E + NO_2^- \rightarrow E.NO_2^-, \quad (1)$$

$$E.NO_2^- \text{ — (oxidation)} \rightarrow E.NO_3^-, \quad (2)$$

$$E.NO_3^- \rightarrow E + NO_3^- \text{ (reaction velocity} = V), \quad (3)$$

$$E.NO_3^- + ClO_3^- \rightarrow E.ClO_3^- + NO_3^- \text{ (reaction velocity} = v). \quad (4)$$

Reaction 4 represents a continuous removal of enzyme from the oxidizing system, and so the overall rate of nitrite oxidation progressively decreases as oxidation proceeds. The velocity ratio v/V has been calculated in a variety of ways from the experimental data. It proves to be proportional to chlorate concentration but to be independent of nitrite concentration or cell density. It is also independent of nitrate concentration. The effect of chlorate on nitrite oxidation thus differs from its bacteriostatic effect which is reversed by nitrate (Lees & Quastel, 1945).

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The Estimation of Serum Mucoproteins. By A. J. Anderson and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

The application of the diphenylamine colour reaction to the estimation of urinary mucoproteins (Anderson, 1954; Anderson & Maclagan, 1954) made it desirable to develop a similar method for the determination of serum mucoproteins. The previous method of Ayala, Moore & Hess (1951) was undesirable for our purpose as we found it to give much higher values than other published methods (Winzler, Devor, Mehl & Smyth, 1948; Simkin, Bergman & Prinzmetal, 1949) probably owing to the inclusion of polysaccharides. However, by combining the diphenylamine reaction with the precipitation technique of Winzler an acceptable method was developed as follows:

To 2 ml. of serum is added 6 ml. of 0·2 M sulphosalicylic acid. The mixture is filtered after 10 min. (no. 50 Whatman) and 2 ml. of 5% (w/v) phosphotungstic acid in 2 N-HCl added to 3 ml. of filtrate. After centrifuging the mucoprotein precipitate is washed with acetone and dissolved in 2 ml. of water at pH 9–9·5. After reprecipitation with 8 ml. of acetone and one drop of saturated aqueous NaCl it is dissolved in 4 ml. of water at pH 9–9·5. After centrifuging, 3 ml. of diphenylamine reagent (1 g.

diphenylamine, 90 ml. glacial acetic acid, 10 ml. conc. H_2SO_4) are added to 3 ml. of the supernatant fluid. After heating for 30 min. at 100° the colour developed is read in a photoelectric colorimeter using a spectrum green filter (520 mμ.). This is compared with the colour produced by a preparation of urinary mucoprotein (Anderson & Maclagan, 1954) treated similarly. A linear relation exists between extinction and amount of urinary mucoprotein up to about 6 mg./2 ml. water.

Unavoidable co-precipitation of serum mucoproteins during the initial deproteinization with sulphosalicylic acid causes low recoveries of added urinary mucoprotein (65–85%) as noted by Winzler (1948). Values obtained are therefore about 30% low. Isolation of serum mucoproteins by this method yielded 70 mg./100 ml. serum with a mobility (paper electrophoresis, pH 8·6) of serum α_1 globulin. Preliminary analysis: N, 7·8%, reducing power before and after hydrolysis (2N-HCl, 100°, 4 hr.), 5·9 and 14·6%. The chromogenic power with diphenylamine was almost identical with that of the urinary mucoprotein standard.

No significant difference was found in the average serum mucoprotein level of twenty-five normal males, $140 \pm 9{\cdot}5$, and twenty-five normal females, $134 \pm 6{\cdot}8$ mg./100 ml.

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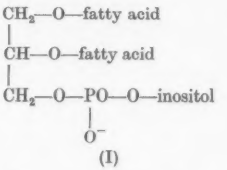
The Ethanol-Insoluble Phosphatides of Mammalian Liver. By J. N. Hawthorne. (*Department of Pharmacology, Medical School, Birmingham* 15)

An ethanol-insoluble liver phosphatide fraction has been prepared by solvent extraction and reprecipitation (cf. Folch, 1949; Folch *et al.* 1951). As obtained from cat and rabbit liver it contains at least two phosphatides, phosphatidyl serine and an inositol-containing phospholipid.

The material was prepared by extracting the fresh liver with 2:1 chloroform–methanol and washing by Folch's method (1951). The washed extract was dried off at 40° *in vacuo*, extracted twice with acetone, and after a further extraction with ethanol, dissolved in a small volume of ether. The white insoluble material was rejected and the extract dried *in vacuo* as before. The solid remaining was precipitated twice from chloroform by two volumes of methanol. After drying, two preparations from rabbit liver had 3·44 and 3·62 per cent phosphorus respectively.

The organic phosphates liberated on acid and alkaline hydrolysis were studied by methods described earlier (Hawthorne & Chargaff, 1954). Three substances were separated by paper chromatography; glycerophosphate, a substance similar in position to inositol monophosphate and traces of a third less soluble phosphate.

No appreciable amount of inositol diphosphate was isolated, but its presence is not excluded. Brain tissue contains an inositide having doubly phosphorylated inositol in its structure (Folch, 1949) and therefore a much higher phosphorus content (7–8%). The chromatographic evidence and P-content reported here suggest that liver contains a monophosphoinositide, but further structural work is not worth while until better methods are developed for the isolation and separation of phosphatides. McKibbin (1954) has reported the presence of an inositide in horse liver, and his evidence also suggests that inositol monophosphate appears in the molecule. A structure (I) like that of the inositol lipid isolated from heart muscle by Faure & Morelec-Coulon (1954) would fit both his results and the present ones. A preliminary report of purifications using the silicic acid column described by McKibbin & Taylor (1952) will appear elsewhere.



(I)

I would like to thank Professor A. C. Frazer for his interest and for providing facilities for this work.

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Free and Fixed Glycogen in Skeletal Muscle of Rats. By ADRIENNE J. M. KITS VAN HEIJNINGEN and A. KEMP (*introduced by* B. MENDEL). (*Pharmaco-Therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland*)

It has been known for many years that part of the tissue glycogen remains undissolved when tissues are extracted with trichloroacetic acid (TCA). This could be due either to artificial fixation of part of the glycogen to insoluble tissue elements by the action of TCA, or to the fact that tissue glycogen appears physiologically in two forms, a TCA-soluble or free form, and a TCA-non-soluble or fixed form. If these two forms are physiological entities it was thought that they would have different functions, and hence behave differently in a physiological process. If, on the other hand, they are artifacts, it was considered unlikely that two different methods of extraction would produce qualitatively and quantitatively the same artifact.

It has recently been demonstrated that all tissue glycogen can be extracted with TCA at 100° (Kemp & Kits v. Heijningen, 1954). By performing the TCA extraction of the tissues in two stages, i.e. with TCA at room temperature and subsequently with fresh TCA at 100° the two glycogen fractions can be determined separately.

The contraction of the gastrocnemius muscle of a rat was chosen to study the influence of a physiological process on both glycogen fractions. The other unstimulated gastrocnemius of the same animal served as control.

The extraction of the skeletal muscle with distilled water containing 0·02N Versene (ethylenediaminetetraacetic acid) to prevent glycogenolysis (Gross, 1953) was chosen as the method of extraction to be compared with the TCA extraction. The Versene extracts were deproteinized with metaphosphoric acid. Glycogen was determined in the tissue extracts with the aid of Mendel's colour reaction (Kemp & Kits v. Heijningen, 1954).

It appeared that muscular contraction reduced the free glycogen proportionally more than the fixed glycogen. Consequently, the ratio free:fixed glycogen decreased in comparison with the control.

The extraction with Versene yielded the same values for both glycogen fractions as did the TCA extraction.

From these results it is concluded that glycogen appears in tissues in two forms, a free and a fixed one. However, they offer no clue regarding the question which physiological function should be ascribed to which fraction.

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Species Differences in the Intercellular Plasma of the Packed Cell Column. By F. W. JENNINGS, I. M. LAUDER and W. MULLIGAN. (*Glasgow University Veterinary School, Glasgow*)

Measurement of the plasma trapped in the packed cell column of the haematocrit has been the subject of numerous investigations (see review by Gregersen, 1951). Although widely different estimates have been proposed by different workers, it is now generally agreed that for human blood, under standard conditions of centrifugation (1500 *g* for 30 min.), about 4–5% of the packed cell column consists of plasma.

In our experiments on the blood of domestic animals we have used a 'direct' procedure similar to that described by Leeson & Reeve (1951), i.e. the addition of serum albumin trace-labelled with ^{131}I to the blood before centrifugation, and analysis of packed cells and supernatant for ^{131}I after spinning. Centrifugation was carried out in polythene tubes (Randolph & Ryan, 1950) and packed cells for analysis were obtained by clamping and cutting off the tube below the top of the red cell column, or by plunging the tube into a solid CO_2–acetone mixture and sectioning the frozen column. The latter method allows the distribution of plasma in the column to be studied.

Using these methods we have found interesting species variations in 'trapped plasma' (Jennings, Lauder, Mulligan & Urquhart, 1954), e.g. under identical conditions the following figures for the plasma content of the red cell column were obtained: rabbit, 3·3%; dog, 3·1%; horse, 2·9%; bovine, 12·5%. Later experiments have shown that sheep blood, like bovine, also shows a high value for plasma in the red cell column under conventional conditions of centrifugation.

Temperature during centrifugation was found to have a marked influence on the magnitude of the 'trapped plasma', particularly in the case of bovine and sheep bloods.

The accuracy of the method used and the implications of the results obtained will be discussed.

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A Possible Source of Error in the Estimation of Haemoglobin by Photoelectric Methods. By H. LEHMANN and HEDY L. KAY. (*Department of Pathology, St Bartholomew's Hospital, London, E.C.* 1)

A method used widely for measurement of foetal haemoglobin is a modification of the technique of alkali denaturation and removal of denatured pigment by precipitation developed by Singer, Chernoff & Singer (1951). The haemoglobin solution is prepared by lysis, removal of lipoids by extraction with toluene and of stroma by centrifugation. In these solutions values for E (1 cm. pathway, $\lambda_{540\,m\mu}$) at rising haemoglobin concentrations are linear only if they are determined by highly precise instruments. We have compared such a precision instrument (SP 500 Unicam) with other types of apparatus. With a defraction grating spectrophotometer (SP 300 Unicam) at 540 mμ, or with a photoelectric colorimeter using a green filter (Ilford 404 or 625) no straight line is obtained when readings are plotted for concentrations above 50 mg. %. In Singer's method the filtrate containing the alkali resistant haemoglobin is compared with a solution containing the 'total' haemoglobin which is prepared by adding 0·1 ml. of a 9–11 g. % haemoglobin solution to 5 ml. of distilled water. Unless a high precision spectrophotometer is used, as was done by these authors, the extinction of light by the control solution may only be half that to be expected if values for E were linear. Unless this is realized, abnormally high levels of alkali-resistant pigment (> 1·7 %) will be observed in normal bloods. These considerations apply also to the measurement of solubility of haemoglobin variants in phosphate buffers by Itano (1953). To a lesser extent they also apply to water haemolysates of red cells. A straight line may not be plotted for E when the haemoglobin concentrations are above 75 mg. %. In the measurement of osmotic fragility of red cells (Dacie, 1950) 0·05 ml. of blood is diluted 100 times. If there is no anaemia, haemoglobin will exceed the concentration of 75 mg.% when more than 50 % of the cells are lysed. In the measurement of oxyhaemoglobin (King, 1951), 0·02 ml. of blood is diluted 200-fold. Therefore only bloods with a haemoglobin percentage below 15 g. will be examined with safety using the less precise instruments.

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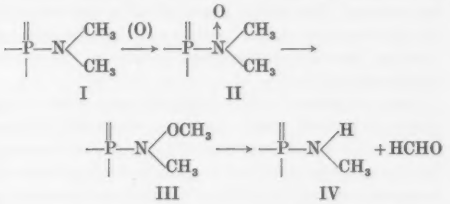
Chemical Oxidation of a Phosphoramide to a Phosphoramide *N*-oxide Anticholinesterase and its Subsequent Rearrangement. By H. TSUYUKI, M. A. STAHMANN and J. E. CASIDA (introduced by F. L. WARREN). (*Departments of Biochemistry and Entomology, University of Wisconsin, Madison, U.S.A.*)

The insecticide octamethylpyrophosphoramide, Schradan, is enzymically oxidized *in vivo* or chemically by permanganate to a highly effective anticesterase (Hartley, 1951; Casida, Allen & Stahmann, 1953; 1954).

Schradan oxidation has also been effected with dichromate, hypochlorite, bromine water and peracetic acid to form products which yield formaldehyde on treatment with acid. The products from hypochlorite and peracetic acid oxidation were separated by column chromatography and shown to be different. The biologically oxidized Schradan and the material from hypochlorite oxidation were more polar than Schradan and inhibited cholinesterase 50 % at 7×10^{-6} M. The material from peracetic acid oxidation was less polar than Schradan and inhibited at 5×10^{-4} M. A transformation of the highly unstable product from biological, permanganate or hypochlorite oxidation was suggested by the solubility changes on purification by column chromatography or countercurrent distribution. Differences in infrared absorption spectra, antiesterase activity, solubility, and mode of chemical decomposition also indicated a structural difference between the initial highly unstable oxidation product and the less reactive transformed product.

The chemical and physical properties of the biological oxidation product indicate that it is the same as that produced by permanganate and is the mono-*N*-oxide of octamethylpyrophosphoramide, in which the oxidized amide group has the structure represented by II. The phosphoramide *N*-oxide readily undergoes a rearrangement similar to that shown by amine oxides to produce the substituted hydroxylamine represented by III. This rearrangement occurs rapidly in aqueous solutions under slightly alkaline conditions, or in anhydrous chloroform on heating, or in the presence of acetic acid. This explains why peracetic acid oxidation produces the substituted hydroxylamine. The rearranged product (III) is more stable and a less

active antiesterase than the phosphoramide *N*-oxide (II), and can be decomposed to formaldehyde and a heptamethylpyrophosphoramide (IV).



This rearrangement may clarify some of the experimental difficulties encountered in studies of phosphoramides. A rearrangement of the *N*-oxide after reaction with the enzyme might also contribute to the prolonged *in vivo* cholinesterase inhibition often occurring with phosphoramide poisoning in animals.

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Chromatography of Glycerophospholipids on Silica-impregnated Paper. By C. H. Lea and D. N. Rhodes. (*Low Temperature Research Station, University of Cambridge, and Department of Scientific and Industrial Research*)

In a previous communication (Lea & Rhodes, 1954) it has been shown that the phospholipids of egg yolk can be fractionated on paper impregnated with silicic acid or on silicic acid columns and pure phosphatidylethanolamine and phosphatidylcholine fractions isolated. In the present work the chromatographic behaviour of a series of individual glycerophospholipids and glycerophospholipid fractions, both natural and synthetic, on silica-paper has been examined.

Phosphatidylcholine and phosphatidylethanolamine fractions were prepared from egg phospholipid by the silicic acid column method, and the corresponding fully saturated materials by subsequent catalytic hydrogenation. The lyso-compounds were prepared by the silicic acid column method from the phospholipids of yolk which had been incubated with rattlesnake (*Crotalus adamanteus*) venom. Phosphatidylserine was prepared from ox brain. To these were added, by the kindness of various workers, dipalmitoleyl-lecithin from yeast (Hanahan & Jayko, 1952), sphingomyelin from ox brain and spinal cord, several synthetic phosphatidylcholines and phosphatidylethanolamines and a synthetic ethanolamine plasmalogen (acetal phospholipid, Egerton & Malkin, 1953). With the exception of one phosphatidylethanolamine all of the synthetic substances were fully saturated.

The best results were usually obtained with 20–30% (v/v) methanol in chloroform. Separations were consistently obtained on different papers and the spots could readily be identified by the use of tracer substances and appropriate reagents, but the R_F values themselves varied considerably from paper to paper.

Phosphatidylethanolamines readily separated from phosphatidylcholines and the lyso-compounds from the corresponding diacyl substances. Plasmalogen and lecithin frequently overlapped but could be distinguished by the ninhydrin spray. Sphingomyelin and lysophosphatidylethanolamine, which also ran close together, were well separated from lecithin and phosphatidylethanolamine and could likewise be distinguished by appropriate reagents. Typical R_F values would be phosphatidylethanolamine 0·85, lecithin and ethanolamine plasmalogen 0·65, lysophosphatidylethanolamine and sphingomyelin 0·5 and lysolecithin 0·3.

Since egg phosphatidylcholine (iodine value 80) and phosphatidylethanolamine fractions travelled with the corresponding hydrogenated materials (iodine value 2) and with the corresponding individual synthetic saturated (C_{14}–C_{16}) substances, and the lecithins also with the individual natural unsaturated (dipalmitoleyl) lecithin, and gave equally compact spots, it was evident that no appreciable separation according to degree of unsaturation or chain length of the constituent fatty acids was occurring.

Subsidiary spots were detected with a number of the substances examined indicating the presence of impurities, possibly due in some cases to decomposition since preparation.

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Electrophoretic Examination of the Effect of Heparin on Serum Lipoproteins. By W. G. Dangerfield and Elspeth B. Smith. (*Department of Chemical Pathology, St Bartholomew's Hospital, London*)

The intravenous injection of heparin clears lipaemic sera as shown by many workers, and causes a redistribution of low density lipoproteins (Graham *et al.* 1951). We have studied its effect on the electrophoretic mobility of the serum lipoproteins in patients who are receiving heparin treatment, and find that both the α- and β-lipoproteins are accelerated (cf. Comfort, 1953, 1954).

In the initial experiments 15000–20000 i.u. of heparin were given intravenously, and the serum collected 20 min. to 2½ hr. later; subsequently, 10 000 i.u. was found to be sufficient, and the standard dose now used is 12000 i.u. The effect is quickly produced, but is short lived—at 13 min. it appears to be fully developed; by 45 min. it is diminishing, and at 2½ hr. the pattern has returned to normal.

Heparin added *in vitro* at 10 times the calculated concentration in the serum produces a faint blurring of the lipoprotein pattern, but no acceleration. If, however, a small proportion of serum, taken from a patient 20 min. after injection of heparin, is added to a control serum, the β-lipoproteins are markedly accelerated. This effect is presumably produced by an 'accelerating factor' which may be the same as clearing factor and is stable *in vitro* over considerable periods.

Two samples of serum from patients with severe diabetic ketosis gave patterns resembling those found after administration of heparin; on recovery the patterns returned to normal. Various substances have been added to serum *in vitro* in an attempt to simulate the heparin effect; they include *Clostridium welchii* lecithinase and surface active substances such as lecithin, bile salts and Teepol. The changes in the patterns resemble to some extent, the heparin effect, particularly in the case of lecithin.

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The Incorporation of ^{65}Zn in Mouse Mammary Tumours. By R. Tupper, R. W. E. Watts and A. Wormall. (*Departments of Biochemistry and Physiology, The Medical College of St Bartholomew's Hospital, London, E.C.* 1)

Conflicting observations have been made about the zinc content of transplanted or induced tumours in relation to that of normal tissues. We decided, therefore, to study the uptake of ^{65}Zn by a spontaneously occurring tumour, following the single intraperitoneal injection of the isotope as a ^{65}Zn-containing zinc glycine complex. The tumour-bearing mice used were of the Strong A, ABC and C3H strains in which there is a high incidence of spontaneously occurring mammary adenocarcinomata amongst the females. Non-tumour-bearing mice of a mixed strain having a negligible incidence of mammary tumours were used as additional controls. In a similar investigation with transplanted mammary gland carcinomata in mice, Heath (1949) and Heath & Liquier-Milward (1950) found that 18 hr. after the injection of ^{65}Zn the carcinomata contained more ^{65}Zn per g. of wet tissue than did the corresponding normal mammary tissue.

In our experiments the mice (sixty tumour-bearing and sixty non-tumour-bearing) were killed in groups at intervals of 3 hr. and 1, 3, 7, 14 and 21 days after the injection of ^{65}Zn. The tissues were digested with H_2SO_4 containing 0·5% SeO_2, and radioactivity measurements made by a scintillation counter as described by Banks, Tupper, Watts & Wormall (1954, 1955), the results being expressed as the amount of ^{65}Zn/mg. tissue N.

A significant difference ($0{\cdot}01 > P > 0{\cdot}001$) existed between the ^{65}Zn contents of the carcinoma tissue and those of the 'normal' (apparently non-malignant) mammary tissues of the same mice. If the values for the mice killed at the 3 hr. interval are not included, $P < 0{\cdot}001$. There was no significant difference between the concentrations of ^{65}Zn in normal mammary gland tissues from the injected tumour-bearing and non-tumour-bearing mice ($0{\cdot}2 > P > 0{\cdot}1$).

The ^{65}Zn contents of the liver, lungs, kidney, skin and blood from the injected tumour-bearing and non-tumour-bearing mice were also studied. The values for the tumour-bearing mice did not differ significantly from those for the non-tumour-bearing animals.

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Extravascular Antibody. By G. E. Francis, J. D. Hawkins and A. Wormall. (*Department of Biochemistry and Chemistry, The Medical College of St Bartholomew's Hospital, London, E.C.* 1)

When an antigenic protein (e.g. bovine serum albumin or horse serum γ-globulin) is injected intravenously into rabbits which had previously received immunizing injections of the same protein, a part of the test antigen is eliminated from the blood stream very rapidly, by a process having a half-life of the order of 5 min., and effectively complete within 45 min.

This rapid process is almost certainly due to a type of *in vivo* precipitin reaction, the precipitated complex being removed from the circulation very rapidly and accumulating in certain tissues, notably liver, spleen and lungs (Francis, Hawkins & Wormall, 1953).

The proportion of the injected test dose eliminated *in vivo* in this manner increases with decrease in the total amount of antigen injected, and the amount removed by the *in vivo* precipitin reaction can usually be determined by following the elimination rate from the blood stream for 1–2 hr. This amount may then be compared with the amount of the same antigen which the total plasma of the immunized rabbit would be capable of precipitating *in vitro*, when treated with the same amount of antigen. In practically every case tested the amount removed *in vivo* was greater than that precipitated *in vitro* and was commonly approximately double; this suggests the presence of extravascular or non-circulating antibody.

This characteristic immune elimination of antigen has been observed in a few cases 2 weeks after a single immunizing injection when no circulating antibodies had appeared. Once circulating antibodies had appeared, the presence of extravascular antibody remained demonstrable 3 weeks after immunization, even when the antibody titre of the serum was falling.

Similar experiments are also being conducted with passively immunized rabbits, in an attempt to clarify the nature of this extravascular antibody.

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Protection of Rabbits against Benzoylcholine by Injection of Pseudocholinesterase. By H. Lehmann and Elsie Silk. (*Department of Pathology, St Bartholomew's Hospital, London, E.C.* 1)

Benzoylcholine resembles succinyldicholine in being a substrate of pseudocholinesterase and not of true cholinesterase. It inhibits the true cholinesterase of man (laked red cells centrifuged and supernatant diluted 1:150, acetylcholine substrate 0·005M) from 30% at a concentration of 0·01M to 85% at a concentration of 0·3M. Benzoylcholine injected intravenously into rabbits will at a dosage of 8–14 mg./kg. body weight produce a head drop lasting 40–120 sec. We have investigated the part played in this response by the plasma pseudocholinesterase level in rabbits. The normal pseudocholinesterase level in rabbits is 10–20 units (determined after McArdle, 1940). Intravenous administration of human pseudocholinesterase raised this level: 5000 units per kg. body weight to 200 units and 10000 units per kg. body weight to 300 units. Immediately following the injection of pseudocholinesterase the rabbits were protected against the effect of benzoylcholine and were quite unaffected by a dosage of benzoylcholine which would normally render them paralysed. This protection lasted with the higher dosage of pseudocholinesterase for at least 1 week and was still partially present in the second week.

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DEMONSTRATION

Immunological Analysis of Proteins by a Gel-diffusion Method. By P. G. H. GELL. (*Department of Experimental Pathology, University of Birmingham*)

The use of antisera raised against human serum proteins, and other complex antigens, for qualitative and semi-quantitative analysis will be demonstrated; the technique is similar to that used by Grabar (1954). Antigen and antibody diffuse towards one another in 0·5% agar in Petri dishes or on slides: a family of lines develops each of which corresponds to an antigen-antibody system at 'optimal proportions'. The gel is then dried down, covered with a plastic coating and mounted as a lantern slide. The method is of value in demonstrating (*a*) the absence of a normal component, e.g. of γ-globulin in congenital α-γ-globulinaemia, (*b*) the complexity of a protein fraction, e.g. β-globulin in human serum, (*c*) the change in proportions of components when fractionating a protein mixture, (*d*) the presence of contaminating antigens in apparently pure protein preparations, and (*e*) progressive changes in the properties of individual proteins, e.g. denaturation. The plate method, moreover, allows of the side-by-side comparison of several different antigen preparations, and of detecting the presence in them of identical components, which give rise to precipitation lines fused at their point of junction.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **335th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, Guy's Hospital Medical School, London, S.E.* 1, *on Friday,* 17 *December* 1954, *at* 2 *p.m. when the following papers were read:*

COMMUNICATIONS

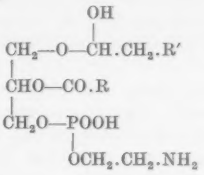
Observations on the Acetalphospholipids of Brain Tissue.

By G. B. ANSELL and JOYCE M. NORMAN. (*Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff*)

It has previously been reported that glycerylphosphorylethanolamine (GPE) obtained by mild acid treatment of the alkali-stable phospholipid fraction of brain tissue probably derives from the ethanolamine-containing acetalphospholipid (Ansell & Norman, 1953). In the present investigation total rat-brain lipid was freed from contaminating water-soluble substances by extraction with aqueous $MgCl_2$; it was then saponified, with aqueous NaOH, neutralized with HCl and treated with trichloroacetic acid by a method similar to that of Schmidt, Benotti, Hershmann & Thannhauser (1946). After separation of water-insoluble material, the sodium chloride and glycerophosphate were removed from the filtrate with suitable cation and anion exchange resins. Paper chromatography of the remaining substances revealed the presence of only two phosphate esters. One had the properties of GPE; the other has been provisionally identified as glycerylphosphorylserine (GPS).

If it is assumed that prolonged saponification breaks down 'classical' ester phosphatides to fatty acids, glycerophosphate and the free base, then the GPE and GPS must derive from acetalphospholipids. The presence of a serine-containing acetalphospholipid in brain tissue has previously been suggested by Klenk & Böhm (1951) and Amelung & Böhm (private communication).

Further experiments were carried out on the GPE eluted from chromatograms after location by the method of Wade & Morgan (1953). Estimation of the phosphate labile to periodic acid gave only approximately 50 % of the phosphate that would be expected if the substance were entirely α-GPE. The properties of the remaining phosphorus compound, which could not be separated chromatographically from the other, agreed with those of β-GPE prepared by the catalytic hydrolysis of a synthetic β-kephalin. The GPE obtained after saponification and acidification of purified ethanolamine 'kephalin' (a mixture of ester- and acetal-phospholipid isolated from brain tissue by the Folch (1942) procedure) behaved similarly. The formation of a mixture of α- and β-GPE from the ethanolamine-containing acetalphospholipid would not be inconsistent with the formula for such an acetalphospholipid recently suggested by Klenk & Debuch (1954):



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Biological Synthesis of Ascorbic Acid: the Conversion of Derivatives of D-Galacturonic Acid into L-Ascorbic Acid by Plant Extracts.

By L. W. MAPSON and F. A. ISHERWOOD. (*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research*)

From our previous studies (Isherwood, Chen & Mapson, 1954), the synthesis of ascorbic acid may be conveniently regarded as occurring in three stages. These, described in terms of the derivatives which will function as intermediates though they may not be those actually formed in the plant, are first, the conversion of galactose into galacturonic acid, second the reduction of the methyl ester of D-galacturonic acid to the methyl ester of L-galactonic acid, and finally the oxidation of the γ lactone of L-galactonic acid to L-ascorbic acid.

The third stage in the synthesis in the plant, the conversion of the lactone of galactonic acid into L-ascorbic acid, has been shown to occur *in vitro* in

the presence of enzymes contained within the mitochondria (Mapson, Isherwood & Chen, 1954). In the present work, the second stage in the synthesis, namely the enzymic conversion *in vitro* of derivatives of galacturonic acid into derivatives of L-galactonic acid has been investigated, and the enzyme mechanism associated with the reduction of the uronic acid to the aldonic acid characterized. This enzyme, which is present in pea seedlings, catalyses a reaction between esters of galacturonic acid and reduced triphosphopyridine nucleotide (TPNH), whereby the galacturonic ester is reduced to a galactonic acid derivative. The evidence, at present available, suggests that this derivative is L-galactono-γ-lactone, although the prior formation of an unstable intermediate is not excluded.

The reduction of the galacturonic ester is limited by (1) the instability of the galactonic acid derivative formed and (2) the reversible inactivation of the enzyme during the progress of the reaction. The reaction may conveniently be studied by using either malic or *iso*citric enzyme systems as the source of TPNH. The enzyme is present in the soluble part of the cytoplasm but is absent from the mitochondria prepared from pea seeds in the early stages of germination.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Estimation of Plasma Cholesterol Fractions by Elution from Paper. By A. COMFORT. (*Department of Zoology, University College, London, W.C.* 1)

The distribution of lipids at paper electrophoresis is usually studied by staining with a Sudan dye. The distribution of cholesterol alone, however, must be plotted by elution and direct estimation. We have found the following method suitable for plotting cholesterol distribution in electrophoretic studies of plasma.

Each run is carried out on five equal samples of plasma or serum (triplicate estimation and two markers: 0·05–0·3 ml. each sample, according to the serum cholesterol concentration). These are run side by side on a sheet of Whatman 3 MM paper by the method of Flynn & de Mayo (1951), in an enlarged tank giving an overall separation of 12–15 cm. The paper is quickly air-dried in a horizontal position at 100°, and the markers stained with azocarmine B and Sudan black respectively. The strips for estimation are marked, separated and cut, either into 10–12 equal sections extending from the γ-globulin boundary to a point at least 1 cm. ahead of the albumin front, or so as to isolate particular protein bands. The cholesterol is extracted from the paper sections with acetone. Each individual section is folded concertina-wise, pushed to the bottom of a test tube and extracted (1) with 7 ml. acetone for 2 min. on a waterbath at 80°, (2) with 5 ml. acetone for 5 min. with shaking at room temperature. Reboiling leads to bumping and does not increase yield. The pooled extracts of each section, with a crumb of porcelain, are taken to dryness on a water bath at 100°. For most qualitative and semi-quantitative studies colour development can be carried out in the same tubes, adding the modified Liebermann–Burchardt reagent of Abell, Levy, Brodie & Kendall (1952) directly to the dried unsaponified residues and plotting optical density/migration distance. The method requires a minimum serum cholesterol level of 50 mg./100 ml. Recovery against total serum cholesterol is about 90 %. Repeatability has been found to depend chiefly on accuracy in applying the serum to the papers. It is comparable to that obtained in other techniques involving extraction, e.g. of dye, from electrophoretic papers.

This work was carried out under a grant of the Nuffield Foundation.

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Amino Acid Conjugates in Decationized Urine. By N. R. LING. (*Area Central Laboratory, Manor Hospital, Bath*)

Urine (100 ml.) was decationized by passage through a column (21 × 2 cm.) of the sulphonated polystyrene resin Amberlite IR 112 (H). Effluent and washings, which were free of amino acids except taurine, were run into a carbonate-free column (30 × 1 cm.) of the strong base resin Amberlite IRA 400 (OH). Amino acid conjugates were recovered from the column with N/6 acetic acid N/12 formic acid (500 ml.) followed by N/4 formic acid (200 ml.) followed by N/12 hydrochloric

acid N/6 formic acid; all solutions contained 10% (v/v) acetone. The fractions (vol. 6 ml.), after evaporation to dryness at 40°, were hydrolysed in 5·7 N-HCl at 110° for 20 hours, and the liberated amino acids identified by paper chromatography after removal of HCl at 40° and desalting. A group (I) removed by acetic acid contained α- and β-alanine, γ-amino-*n*-butyric acid, aspartic and glutamic acids, glycine, proline, sarcosine, serine, and valine; two fractions rich in conjugated β-alanine and γ-amino-*n*-butyric acid respectively were obtained from this group by elution analysis employing Amberlite IRA 400 (acetate)/0·1 N acetic acid in 10% acetone. A group (II) displaced by formic acid contained α-alanine, glutamic and aspartic acids, glycine, serine, threonine, sarcosine, proline, valine, leucine and isoleucine. Two fractions (III and IV) containing conjugated forms of glycine and glutamic acid respectively were eluted by formic acid. A group (V) displaced by HCl contained α-alanine, aspartic and glutamic acids, glycine, valine, tyrosine, and leucine.

Conjugates from groups II, III and IV were partially extractable with ethyl acetate. Treatment of the decationized urine with decolourizing charcoal (2 g./100 ml. urine) removed part of group II, most of groups III, IV and V, and 40% of group I conjugates; adsorbed conjugates could be recovered from an aqueous suspension of the charcoal by treatment with 25% (v/v) benzyl alcohol in acetone (16 ml./100 ml. urine).

A Bile Alcohol from Cyprinidae. By G. A. D. Haslewood. (*Guy's Hospital Medical School, London, S.E.* 1)

It has already been reported (Haslewood, 1951) that the chief bile salt in the carp, *Cyprinus carpio*, was found to be a neutral substance conjugated with sulphate. The same has now been shown to be true of other Cyprinidae (roach, chub, tench). The bile alcohol has been isolated as its tetraacetate, probably $C_{27}H_{44}O(OCOCH_3)_4$, double m.p. about 107–108° and 136–137°. This substance was obtainable in a yield of about 20% after acid or alkaline hydrolysis of the crude bile salts, when sulphate very approximately corresponding to the splitting of a pentahydroxy alcohol sulphate, $C_{27}H_{43}(OH)_4 \cdot OSO_3^-$, was isolated as $BaSO_4$. It is suggested that this bile alcohol, 'cyprinol', may be characteristic of Cyprinidae bile and that it represents an evolutionarily 'primitive' character of the species in which it occurs. The new tetraacetate somewhat resembles, but is different from, ranol tetraacetate from frog bile (Haslewood, 1952) and separates readily from the latter substance on paper chromatograms.

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Partially Synthetic Trihydroxycoprostanic Acid. By R. J. Bridgwater (introduced by G. A. D. Haslewood). (*Guy's Hospital Medical School, S.E.* 1)

A 3α:7α:12α-trihydroxycoprostanic acid, $C_{27}H_{46}O_5$, m.p. 172°, has been isolated from the bile of the frog *Rana catesbiana* and of Crocodilydae. Its carbon skeleton was shown to be that of coprostane (Bridgwater & Haslewood, 1952). The acid m.p. 172° from the bile of *R. catesbiana* was called 'α-trihydroxycoprostanic acid', to distinguish it from a 'β'-compound, m.p. 195–6°, from *R. nigromaculata nigromaculata* (Komatsubara, 1954). The α-acid was also found in this species.

Bile acids with extended side chains were prepared by Kolbe electrolysis with cholic acid and the appropriate half-ester. By this means, homo- and bishomo-cholic acids have been made, and the method was examined for the preparation of trihydroxycoprostanic acids. This involved the electrolysis of cholic acid with the substance $HO_2C.CH_2.CH(Me).CO_2Me$ (I), which possesses an asymmetric carbon atom, and should therefore give rise to two trihydroxycoprostanic acids, isomeric at C-25. The product (*A*) from electrolysis with racemic (I) melted at about 160°, with appreciable softening at 100°. (I) prepared from L-methylsuccinic anhydride gave an acid (II) of m.p. 192–194°, but use of (I) prepared from D-methylsuccinic anhydride gave a product (*B*) with properties (m.p. and optical rotation) similar to those of (*A*).

Acid (II) did not depress the m.p. (194–195°) of a specimen of partially synthetic β-trihydroxycoprostanic acid (III) (Komatsubara, 1954) kindly supplied by Prof. T. Kazuno, and the infrared spectra (comparisons kindly made by I. D. P.

Wootton and H. Wiggins) of (*A*), (II), (III) and (*B*) differed only slightly, the differences being of an order explicable by variations in purity. β-Trihydroxycoprostanic acid would therefore appear to be related to L-glyceraldehyde (cf. Ställberg-Stenhagen & Stenhagen, 1947).

It would thus appear that there are two naturally occurring trihydroxycoprostanic acids, isomeric at C-25. The acid of m.p. 172° (Japanese α-) has already been formulated as trihydroxycoprostan-26-oic acid (Haslewood, 1952), and the acid of m.p. 195–196° (Japanese β-) can therefore be called trihydroxycoprostan-27-oic acid.

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Oxidation–Reduction Properties of Adrenocorticotrophic Hormone. By MARGARET L. DEDMAN, T. H. FARMER and C. J. O. R. MORRIS. (*Department of Experimental Biochemistry, London Hospital Medical College, London, E.* 1)

Kuehl, Meisinger, Brink & Folkers (1953) have reported that adrenocorticotrophic hormone (ACTH) can be oxidized by the action of Fe^{3+} and O_2 to yield a biologically inactive product, which can be reactivated by treatment with H_2S. We have studied the oxidation–reduction properties of the hormone further using concentrates prepared by the oxycellulose process of Astwood, Raben, Payne & Grady (1950) and the adrenal ascorbic acid depletion bioassay of Sayers, Sayers & Woodbury (1949). ACTH may be oxidized to a biologically inactive material by the action of H_2O_2 at room temperature. The optimum pH for the process is 7–7·5. Reaction at a lower pH is slower. While use of a higher pH or of more vigorous oxidizing agents results in irreversible inactivation of the hormone. Peroxide-inactivated ACTH may be reduced to a biologically active material by sulphydryl compounds. The optimum conditions for the reduction are with cysteine hydrochloride at pH 1·9 at 80° for 18 hr. Under these conditions the biological activity is completely restored and in some cases may be higher than that of the starting material. Thioglycollic acid and BAL are also effective in the reduction, while H_2S, ascorbic acid, KBH_4, Zn and HCl and electrolytic reduction are ineffective.

Using these methods, attempts have been made to identify the nature of the oxidation–reduction centre. Amperometric titration indicates that such preparations contain less than 0·03% of SH. Moreover the biological activity is unaffected by treatment with *p*-chloromercuribenzoate. Raney nickel desulphurization of these ACTH preparations does not affect the biological activity, although the methionine present has been removed by the treatment. These observations rule out the participation of sulphur compounds in the oxidation-reduction process. Treatment of oxidized ACTH with dimedon and other carbonyl reagents does not prevent the reduction to a biologically active form, thus making it unlikely that the oxidized form is a carbonyl compound. Oxidation does not alter the u.v. absorption spectrum. Iodination at pH 6·5 does not prevent oxidation or reduction. Alkaline treatment of oxidized ACTH does not prevent reduction. Reaction of oxidized and untreated ACTH with *p*-dimethylaminobenzaldehyde and HCl results in irreversible inactivation suggesting that the 2 position of the tryptophan residue in oxidized ACTH is unchanged. Oxidation methods leading to destruction of tyrosine and tryptophan or to hydroxylation in the aromatic rings invariably led to irreversible inactivation. It is suggested that the oxidation-reduction properties of ACTH may be due to a hitherto unidentified grouping.

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The Nucleic Acid Content of the Seminal Vesicle of the Guinea Pig. By G. F. HUMPHREY and G. M. KELLERMAN. (*Department of Biochemistry, University of Sydney, Australia*)

Some respiratory characteristics of sub-cellular fractions of the seminal vesicle have been studied previously (Humphrey & Robertson, 1953) and pentose and deoxypentose analyses were made to assist in evaluating the cytological purity of the preparations. The analytical results suggested that the seminal vesicle was richer in deoxyribonucleic acid (DNA) than in ribonucleic acid (RNA). This question has now been re-investigated with more accurate techniques.

The results show that the extinction coefficients per mole P per litre at pH 7·2 and 260 mμ. and 1 cm. light path were 10 230 for seminal vesicle RNA and 9240 for DNA. For males kept in separate cages, the RNA.P was 40–60 mg./100 g. of gland and the DNA.P was 14–22 mg. When males were kept for one month in a pen with several females, the glands were smaller and although the RNA.P concentration was as above, the DNA.P rose to an average of 23·5 mg./100 g. and the ratio fell as low as 1·5. These changes in composition are due principally to alterations in the mucous membrane and not the muscular part of the gland. Thus the mucosa from breeding animals had less cytoplasm and double the concentration of DNA.P as compared with segregated males. In castrated animals the total gland weight fell to about a quarter of the normal value and the DNA.P and RNA.P both reached a value of about 30 mg./100 g.

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A Recording Dielectrometric Method for Column Chromatography of the Neutral Amino Acids. By R. A. Grant. (*Department of Biochemistry, The London Hospital Medical College, London, E.* 1)

Conventional methods of column chromatography involve the collection and subsequent analysis of many small fractions of the effluent. In the present method an attempt has been made to cut down the labour involved by continuously analysing the effluent and automatically recording the results.

As a basis for a continuous flow method it seemed convenient to use the effect of neutral amino acids on the dielectric constant of the solution. The molar increments are of the order of 20–25 (Cohn & Edsall, 1943). The large effect is due to the existence of zwitterions in the solution.

An electronic measuring unit has been developed which gives a d.c. output directly proportional to the change in dielectric constant and having a sensitivity of about 100 volts/$\mu\mu$F.

The cell consists of a flattened bulb containing two platinum plates (1 cm.^{2} in area separated by 0·4 cm.) which function as a condenser, the effective volume of the cell is about 0·5 ml. The cell is rigidly mounted and is surrounded by a water jacket through which thermostat water is circulated. As the effluent from the column passes through a cell any change in the dielectric constant is immediately shown on the potentiometer recorder (Elliott, 10 mv), hence as the amino acid bands emerge from the column the recorder traces a series of peaks.

So far the method has been applied to chromatography on cellulose powder columns (Whatman Cellulose Powder and Solka-Floc) 30 × 2 cm. in size. The solvent has usually been a mixture of *n*-propanol and water (15–30 % water).

Before recording separations the column is equilibrated by passing 250 ml. of solvent through it. The measuring unit is also allowed to run overnight to establish electrical and thermal equilibrium. The amino acid mixture for analysis should be essentially free from salts. The mixture is dissolved in a small quantity of water and sufficient propanol added to give the same composition as the solvent. This volume (usually about 2 ml.) is added to the top of the column and allowed to sink in, more solvent is then added and the reservoir connected. The flow rate has usually been 10–15 ml. per hour and the recorder has been set at 1 inch/hour.

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The Hexosamine Ratios in Blood Group Substances. By C. J. M. Rondle and W. T. J. Morgan. (*Lister Institute, London, S.W.* 1)

The recognition by Aminoff & Morgan (1948) of the presence of glucosamine and galactosamine in substantially homogeneous preparations of human blood group substances led to the determination of the amounts of each of these hexosamines in the acid hydrolysis products of the group materials. The method first used (Annison, James & Morgan, 1951) which involved the conversion of the hexosamines into *N*-2:4-dinitrophenyl derivatives and their chromographic separation on a column of Hyflo Super-cel was abandoned in favour of Gardell's simple procedure (Gardell, 1953) which brought

about a complete and practically quantitative separation of the hexosamine hydrochlorides on a column of the cation exchange resin. Dowex 50.

In our experiments mixtures of glucosamine and galactosamine hydrochlorides (total hexosamine 150–400 μg.) were separated on a column (45 × 0·6 cm.) of Zeocarb 225 (200–400 mesh) using 0·34N-HCl for elution. Authentic sugar mixtures (glucosamine-galactosamine ratios varying from 1:1 to 1:5) gave yields of about 99 % (93–102 %) for glucosamine and about 94 % (86–104 %) for galactosamine.

Blood group substances were hydrolysed with 0·5N-HCl to give maximum hexosamine values and the hexosamines were similarly separated on a Zeocarb column and estimated by a modification of the method of Elson & Morgan (1933). The efficiency of the column to separate quantitatively known hexosamine mixtures was repeatedly checked.

Group *A* group substances gave a mean hexosamine recovery of 95 % (90–100 %) and glucosamine-galactosamine ratios from 0·7 to 1·6. Two preparations of *B* substance, suitable for study, gave a recovery of 95 % and 98 % and a hexosamine ratio of 1·7 and 3·5. 'Lewis' (*Le*^{a}) group substance gave a ratio of 3·2 with a 99 % recovery of hexosamines. The ratios found for the group *H* substances examined (8 specimens) varied considerably. The yields of hexosamines were between 84–98 % of the total present and the glucosamine/galactosamine ratios found were between 2·6 and 12·7. The wide variation in ratios found may be related to the very different serological characters of the *H* substances. Leskowitz & Kabat (1954) have recently given hexosamine ratios for human and animal group substances which are in general agreement with our earlier and present values for *A* and *B* substances.

The glucosamine content of the group substances falls in most specimens between 16 and 20 %. The galactosamine value is similar to the glucosamine value in *A* substance but to rather less than 10 % in *B* and *Le*^{a} substances. For certain preparations of *H* substance the galactosamine value falls to about 2 %.

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The Detection of Steroid 17:20-diols on Chromatograms. By I. E. Bush (introduced by I. H. Muir).
(*Medical Unit Laboratory, St Mary's Hospital, London, W.* 2)

Buchanan, Deker & Long (1950) used lead tetra-acetate and sodium metaperiodate–Schiff reagent to detect non-reducing sugars on paper chromatograms, and Marrian (1953) suggested the use of the former reagent for detecting steroid diols. In their original form these methods were unsuitable for steroid work. Since 17:20-diols yield 17-keto-steroids with these oxidants a combination with the Zimmermann reaction (Zimmermann, 1935) was investigated. The methods evolved finally were:

(1) The chromatogram is dipped through 0·4 % $Pb(OAc)_4$ in glacial acetic acid, hung 15 min. at room temperature, and then dried with lamps. It is then dipped through 1 % *m*-dinitrobenzene in ethanolic N-KOH and heated to dryness.

(2) The chromatogram is treated with 1 % HIO_4 in 75 % ethanolic H_2SO_4 (0·4N), hung 30 min. at room temperature, dipped in 0·1N-NaOH, and dried with lamps. The Zimmermann reagent is then applied.

Both methods are reliable and sensitive (1–2 μg./cm.) with 17:20-diols and 17:20:21-triols. Other C_{21} steroids are not detected but reactions with non-steroid substances are to be expected and a control chromatogram treated with *m*-dinitrobenzene alone is necessary. HIO_4 gives a cleaner background colour. The method can be used with chromatograms of urine extracts.

17α:20β-Diols react more slowly than their 20α-epimers and the procedures described are designed for the former compounds.

Three compounds of this type are conspicuous on chromatograms of urine extracts; minor components have not been investigated yet. One is pregnan-3α:17α:20α-triol (Cox & Marrian, 1953), the others appear to be pregnan-3α:17α:20,21-tetrol-11-one and the related 11β-ol. Thus they yield on oxidation with bismuthate (Norymberski, 1952) testan-3α-ol-17-one, testan-3α-ol-11,17-dione, and testan-3α:11β-diol-17-one respectively. The first has an R_F value identical with that of pregnan-3α:17α:20α-triol, while the latter have extremely low R_F values in benzene–aqueous methanol systems (Bush, 1952) and release formic acid on oxidation with metaperiodate (Buchanan, *et al.* 1950). No other structures satisfy all these properties but identification of the latter compounds must await isolation.

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Separation and Purification of Ribonucleic Acid (RNA) from Rat-Liver Extracts. By O. Bassir. (*Area Pathology Laboratory, Westwood Hospital, Beverley*)

The supernatant after the precipitation, by the method of Schmidt & Thannhauser (1945), of deoxyribonucleic acid from the total acid-insoluble fraction of liver extracts consists of breakdown products of ribonucleic acid and phosphoprotein.

Because of the high specific activity of the inorganic phosphate, derived from phosphoprotein (Davidson & McIndoe, 1949), with which ribonucleic acid may be contaminated during separation by this method, an attempt was made to find out how effective was the method of Delory (1938), in separating ribonucleic acid from phosphoprotein phosphorus, in terms of radioactivity.

In the presence of interfering substances, Delory (1938) states that as little as 0·01 mg. of inorganic phosphorus can be precipitated, by entrainment with 'light' magnesium carbonate, as $Ca_3(PO_4)_2$.

Two 5 ml. samples of an aqueous solution, containing 0·5 g. Na_2HPO_4 per litre, and being considerably radioactive (40–60 counts per second) through the addition of carrier-free ^{32}P, were treated by the method of Delory (1938) for the precipitation of inorganic phosphate. Similar samples were subjected to a modified form of the same method, that is, standing the samples overnight in a refrigerator, after the addition of light magnesium carbonate. The amount of radioactive material left unprecipitated in the filtrates, was determined on the following day. The phosphorus content of the precipitate was estimated, in each case. The percentage activity, in duplicate, was 87·0, 91·2 with the standard technique, and 94·7, 93·6 with the modification. These were chemically equivalent to phosphorus percentages of 95, 95, and 99·9, 97·9, respectively.

By using carrier phosphate with Kursannov's (1938) magnesia reagent, the precipitation of radioactive inorganic phosphate was greatly improved; the % activity of the precipitates being 96·1, 96·3. In preparing the carrier phosphate, 20 mg. $Na_2HPO_4, 12H_2O$ is boiled in 5 ml. of water with magnesia mixture and excess ammonia. The product is then allowed to stand for one hour, and centrifuged. To this precipitate is added 10 ml. of the solution containing inorganic phosphate. This is made slightly alkaline, with drops of concentrated ammonia, before the addition of 3 ml. more of the magnesia reagent, and standing overnight in the refrigerator. On centrifuging, a supernatant containing practically no radioactivity is obtained.

Next, both the modified Delory method and the new procedure were applied to radioactive N-NaOH extracts of rat liver with initial counts of 46·0 and 43·0/sec. The recoveries were: modified Delory technique, 93·1; present technique, 98·9.

This work was done during the tenure of a Nuffield fellowship at the Medical Research Council's Human Nutrition Unit.

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Absorption at 275 mμ. in Rat-Liver Fat. By R. J. Ward and T. Moore. (*From the Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council*)

A band at 275 mμ., with other maxima or inflexions at 322, 283 and 332 mμ., was observed by Lowe, Morton & Harrison (1953) in the unsaponifiable fraction of the liver fat of rats deficient in vitamin A. It was suspected that this absorption might be due to abnormal steroid products. The wavelength 275 mμ. is also characteristic of vitamin A epoxide (Karrer & Jucker, 1954) which was earlier described as hepaxanthin (Euler, Karrer & Zubrys, 1933). Absorption at 275 mμ., ascribed to oxidation product of vitamin A, was reported by LePage & Pett (1941) in the blood of humans who had been dosed with the vitamin. Moore & Rajagopal (1939) also noticed a band at 275 mμ. in ether extracts of the livers of rats which received enough vitamin A for growth, but not for storage. The band was lost

during saponification. The present experiments were undertaken to obtain more information on this alkali labile spectrant.

In repeated examinations of ether extracts of livers of rats deficient in vitamin A, a band at 272 mμ. was regularly observed. Saponification of the fat with hot ethanolic KOH caused the band to disappear, but it survived the same treatment when applied direct to the liver tissues. Presumably the tissues protected against oxidation. The band disappeared from solutions which were treated with 85% H_2SO_4, were aerated, or even stored for 37 days at $-10°$; it survived treatment with digitonin. The sterol-free unsaponifiable matter was neither fluorescent under u.v. radiation nor chromogenic in the $SbCl_3$ test. On alumina it was strongly adsorbed, and was eluted with 10% ethanol in light petrol with some destruction. In chromatography on 5% paraffin-coated paper it was also strongly adsorbed, absolute ethanol was necessary for development. Of two fractions one had a single sharp band at 275 mμ. (E 275/E 250 = 1·75). The other fraction combined a band at 275 mμ. with an inflexion at 330 mμ. Both fractions were faintly yellow, but showed no selective visible absorption. Bands at 272–275 mμ. were also found in liver extracts from a rat which had been cured of vitamin A deficiency, from normal sexually immature rats, from a normal pig and guinea pig, but not from a calf.

Absorption at 275 mμ. was also observed in a fraction separated by chromatography on alumina from rats' liver, rich in vitamin A, which had been freeze-dried and stored. The substance responsible, probably an oxidation product of vitamin A, differed from the spectrant already described in being more stable to saponification, and in showing blue fluorescence under u.v. irradiation.

These findings justify no comment on the interesting suggestion of Lowe *et al.* that absorption at 233, 275, 283 and 332 mμ. may be indicative of avitaminosis A. The presence in the liver fat of both normal and deficient animals of an alkali-labile substance with a single band at 275 mμ., however, must be recognized as a complicating factor in studies in this field.

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The Use of Cyanate and Chlorate in Studies on the Relation between the Nitrite Oxidation and the Reduction of a Cytochrome System in *Nitrobacter*. By H. Lees and J. R. Simpson. (*Department of Biological Chemistry, University of Aberdeen*)

It was shown at the last meeting of the Society that chlorate causes a progressive decrease in the rate of nitrite oxidation by suspensions of the autotrophic nitrifying organism, *Nitrobacter*. Evidence was presented for an interaction between chlorate and an intermediary substance which exists at some stage during the cycle whereby nitrite is oxidized to nitrate.

Cyanate, NCO^-, has since been found also to inhibit the nitrite oxidation of resting suspensions of *Nitrobacter*, but in a way different from chlorate. The cyanate-inhibited cells gave a constant reduced rate of nitrite oxidation, independent of their time of incubation with cyanate. Cells inhibited with chlorate could not be reactivated by washing; cyanate inhibition was reversed by washing. Other results support the view that cyanate and chlorate inhibit at different points during the nitrite oxidation, the cyanate inhibition occurring at an earlier stage than the chlorate inhibition.

Three cytochrome bands have been detected spectroscopically in dithionite-reduced suspensions of *Nitrobacter*. The most intense absorption peak was observed at 551 mμ., another at 589 mμ. and a less intense, diffuse band surrounding 522 mμ. An aqueous extract of cells crushed in the Hughes press (Hughes, 1951) gave an absorption band at 551 mμ. on dithionite reduction, but the 589 mμ. band was missing.

The three bands appeared on adding nitrite to a washed suspension of cells at room temperature. They did not appear on adding nitrite in the presence of excess cyanate, but could then be produced by adding dithionite. During aerobic incubation of cells with nitrite and chlorate, there was a gradual fading of the reduced cytochrome band at 551 mμ., leaving the one at 589 mμ. in a much greater relative intensity. After 1 hr. incubation at 25° with chlorate-nitrite, the characteristic intense absorption at 551 mμ. could not be restored by adding either nitrite or dithionite.

It appears likely from the above and related results that there is a significant connexion between the action of cyanate and of chlorate on the rate of nitrite oxidation on the one hand and their respective apparent inhibitions of cytochrome reduction on the other.

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DEMONSTRATIONS

Identification of the Opium Alkaloids. By E. G. C. Clarke and Margaret Williams. (*Royal Veterinary College and Hospital, London, N.W.* 1)

An Internal Standard Flame-photometer for Determination of Calcium in Serum. By R. W. R. Baker. (*Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.* 1)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **336th Meeting** *of the Biochemical Society was held in the Physiology and Biochemistry Department, King's College, Strand, London, W.C. 2, on Saturday, 15 January 1955, at 2 p.m., when the following papers were read:*

COMMUNICATIONS

The Metabolism of 2-Naphthylamine in Rat Tissue Slices. By JOAN BOOTH, E. BOYLAND and D. MANSON. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3*)

The metabolism of 2-naphthylamine and some derivatives has been further investigated by incubating rat liver and kidney slices at 38° in Warburg flasks containing a solution of substrate dissolved in Krebs–Ringer phosphate solution. Glucose was omitted from the Ringer solution because aromatic amines readily formed *N*-glucosides when incubated in the presence of glucose.

The metabolites formed were separated and identified by paper chromatography and colour reactions. All reference compounds were prepared synthetically or biosynthetically. The rate of formation of some of the metabolites was estimated colorimetrically by elution of the appropriate spot from chromatograms run on 3MM paper.

The incubation of approximately 300 mg. tissue in 3 ml. substrate (2×10^{-4}M) dissolved in Ringer solution showed that:

(1) 2-Naphthylamine was converted by liver into about equal amounts (0·7 μmole/g. dry weight tissue/hr.) of 2-amino-1-naphthyl sulphuric acid and 2-amino-6-naphthyl sulphuric acid but some of the 6- derivative was acetylated.

(2) 2-Acetamidonaphthalene was deacetylated by liver and kidney and oxidized to 2-acetamido-6-naphthyl sulphuric acid (0·8 μmole/g./hr.), and to a lesser extent to 2-amino-1-naphthyl sulphuric acid and 2-amino-6-naphthyl sulphuric acid.

(3) Both liver and kidney deacetylated 2-acetamido-1-naphthyl sulphuric acid and acetylated 2-amino-6-naphthyl sulphuric acid.

(4) Liver synthesised sulphuric acid and glucosiduronic acid conjugates from 2-amino-6-hydroxynaphthalene, 2-acetamido-6-hydroxynaphthalene and 2-acetamido-1-hydroxynaphthalene.

(5) Kidney hydrolysed 2-acetamido-6-naphthyl sulphuric acid and 2-amino-6-naphthyl sulphuric acid to the corresponding aminonaphthol but had no action on 2-amino-1-naphthyl sulphuric acid.

Oxidation of 2-naphthylamine with an ascorbic acid-ferrous sulphate reagent (Udenfriend, Clark, Axelrod & Brodie, 1952; Brodie, Axelrod, Shore & Udenfriend, 1954; Udenfriend, Clark, Axelrod & Brodie, 1954), gave 2-amino-1-hydroxynaphthalene and 2-amino-6-hydroxynaphthalene. 2-Acetamidonaphthalene was converted into 2-acetamido-1-hydroxynaphthalene and 2-acetamido-6-hydroxynaphthalene by this reagent, showing that substitution had occurred in the same positions as in the biological oxidations.

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The Enzymic Hydrolysis of Derivatives of Aminophenols. By E. BOYLAND, D. MANSON, P. SIMS and D. C. WILLIAMS. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3*)

Aromatic amines are oxidized in the animal body to *o*- and *p*-aminophenols. 2-Naphthylamine is oxidized by rat liver to 2-amino-6-hydroxynaphthalene and the carcinogenic 2-amino-1-hydroxynaphthalene. These aminonaphthols are rapidly converted to sulphuric esters or glucuronide ethers and excreted as such conjugates in urine. As both human and animal urines contain sulphatase and β-glucuronidase (Boyland, Wallace & Williams, 1954), the aminophenols would be expected to be liberated from their corresponding conjugates in urine. 2-Amino-1-naphthyl sulphuric ester, however, was not hydrolysed by the sulphatases of rat kidney, takadiastase or human urine. Of a series of 18 *o*-aminophenyl sulphuric esters tested, 5 remained unhydrolysed on incubation with takadiastase or with a sample of human urine with high sulphatase activity. The sulphuric esters resistant to

hydrolysis were derivatives of 2-dimethylaminophenol; 4-amino-3-hydroxydiphenyl; 2′-chloro-4-dimethylamino-3-hydroxystilbene; 2′-methyl-4-dimethylamino-3-hydroxystilbene, and 2-amino-1-hydroxynaphthalene; the last four of these aminophenols are either carcinogenic themselves or are derivatives of carcinogenic amines. Most of the sulphuric esters of *o*-aminophenols (esters of *o*-aminophenol, *o*-methylaminophenol, 3-hydroxyanthranilic acid, 2-hydroxysulphanilic acid, 6-amino-*m*-cresol, 2-amino-*m*-cresol, 2-amino-*p*-cresol, 3-hydroxy-4-aminobenzoic acid, 2-amino-4:5-dimethylphenol, 4-dimethylamino-3-hydroxyazobenzene, 1-amino-2-hydroxynaphthalene and 3-hydroxykynurenine) were very slowly hydrolysed but 2-amino-4-chlorophenyl sulphuric ester was hydrolysed moderately rapidly. 3-Indoxyl sulphuric ester was only slowly hydrolysed by sulphatase. All these esters appear to be easily hydrolysed in N-HCl at 100°.

In contrast to the resistance of 2-amino-1-naphthyl sulphuric ester to enzymic hydrolysis, the 2-amino-1-naphthyl phosphoric ester is hydrolysed by phosphatase and both 2-amino-1-naphthyl glucosiduronic acid and 2-acetamido-1-naphthyl glucosiduronic acid are hydrolysed by β-glucuronidase.

Cancer of the bladder is induced in men and in dogs exposed to 2-naphthylamine. When implanted in the bladder of mice, 2-naphthylamine does not induce cancer, but 2-amino-1-hydroxynaphthalene does (Bonser, Clayson, Jull & Pyrah, 1952). The actual carcinogenic agent is probably therefore 2-amino-1-hydroxynaphthalene released from conjugates in urine. The present experiments show that the carcinogenic aminonaphthol would not be liberated from 2-amino-1-naphthyl sulphuric ester but would be produced by enzymic hydrolysis of 2-amino-1-naphthyl glucosiduronic acid. This latter compound is therefore probably an essential intermediate metabolite in the induction of bladder cancer with 2-naphthylamine.

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The Estimation of Carbohydrate in Skin by the Method of Kemp & Van Heijningen. By W. K. Berry and J. R. Burt. (*Chemical Defence Experimental Establishment, Porton, Wilts*)

Kemp & Van Heijningen (1954) described a procedure for estimating glucose and glycogen in the same tissue sample, using the colour reaction with concentrated sulphuric acid as revised by Mendel, Kemp & Myers (1954). The simplicity of the procedure was attractive, and it was desired to use it in studies of the metabolism of skin. We found a slightly different maximum absorption and a slightly different relation between colour intensity and glucose concentration than those given by Mendel *et al.* (1954), but we were able to obtain excellent replicates in practice runs on rat liver. However the method failed when applied to skin because of the presence of relatively large amounts of interfering substances which not only produced deep pink colours before the addition of sulphuric acid, but gave brown colours with it, and depressed the intensity of absorption at the wavelength of 520 mμ. at which measurements would normally be carried out.

Although it is virtually impossible to grind fresh or lipid-free dry skin, a preliminary 5-min. boil in any aqueous solution enables grinding to proceed easily. This yields gelatin; and some trials with commercial powdered gelatin suggested that this was not the interfering substance.

This work was carried out by J.R.B., an undergraduate of St Andrew's University, during tenure of a vacation appointment. We are indebted to the Chief Scientist, Ministry of Supply, for permission to communicate these results. Thanks are due to Cpl. A. G. Whyborn, R.A.M.C., for a few additional data.

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The Presence of Other Substances in Crystalline 'Oestriol'. By Margaret I. Stern and Hannelore Braunsberg. (*University College Hospital Medical School, London, W.C.* 1)

Partition chromatography of several oestriol samples by the method first described by Stern & Swyer (1952), followed by fluorimetry (Finkelstein, Hestrin & Koch, 1947; Braunsberg, Osborn & Stern, 1954) revealed the presence of other highly fluorogenic substances. The present report, though incomplete, may be of some help to others using oestriol prepared from natural sources. Further-

more, the ultimate isolation of these compounds may be of physiological interest.

Using larger chromatography columns consisting of 6·25 g. Celite 535 and 5 ml. 3·1N-NaOH, and 3:17 butanol–chloroform as moving phase, two components were eluted before the oestriol-16α:17β, fraction. The faster one, X_1, gave a green fluorescence similar to that of oestrone and oestradiol on heating with phosphoric acid. The slower one, X_2, is either considerably less fluorogenic, or present in extremely small amounts. Its fluorescence could not be seen, even when over 2 mg. of 'oestriol' were put on the column; but our sensitive fluorimeter (see Braunsberg, Osborn & Stern, 1954) recorded fluorescence in several fractions.

A few other substances were tested on our columns. Oestriol-16β:17β recently isolated from pregnancy urine (Marrian & Bauld, 1954) runs faster than the 16α:17β isomer and may be identical with our substance, X_2. On heating with phosphoric acid, it gave a green fluorescence with a pink colour, the visible absorption spectrum of the solution being identical with that of oestriol-16α:17β. 16-Oxo-oestradiol (also kindly supplied by Prof. Marrian) ran more slowly than oestriol. Its fluorescence on heating with phosphoric acid resembled that of oestrone and oestriol and did not show a pink colour (as do the two oestriol isomers tested).

A carefully recrystallized sample of oestriol prepared by Prof. Marrian did not appear to contain the faster running component X_1.

Since the 16α:17β and 16β:17β isomers of oestriol can be separated on our columns, our results indicate that no rearrangement of one to the other occurs in the 3·1N-NaOH present. Similarly, it can be concluded that our substances X_1 and X_2 are not artifacts arising from any decomposition of oestriol on our columns.

We have some evidence that the substance X_1 is present in unhydrolysed pregnancy urine, indicating that it may not be an artifact formed during hydrolysis.

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Studies on the Use of Sulphonated Cation Exchange Resins for the Hydrolysis of a Mucoprotein (Ovomucoid). By A. S. Dixon. (*Lovett Memorial Laboratories, Massachusetts General Hospital, Boston, U.S.A.*)

Sulphonated cation exchange resins act as insoluble strong acids and have been used as hydrolysing agents for proteins (e.g. Underwood & Deatherage, 1952), polysaccharides (e.g. Deuel, Selnis, Angas-Weisz & Huber, 1951) and reticulin (e.g. Glegg, Eidinger & Leblond, 1953). The present investigation studied the suitability and advantages of these resins for the hydrolysis of mucoproteins, using ovomucoid as a model.

Six sulphonated cation exchange resins in the acid phase were compared for stability in water at 100°. All liberated some H_2SO_4 and a brown material after prolonged heating. The latter was absorbable by charcoal. For Dowex-50 resins, breakdown increased with the degree of cross linkage.

A series of tubes were prepared each containing 4·5 mg. of ovomucoid (Lineweaver & Murray, 1947). 900 mg. of a preparation of Dowex-50, which did not liberate significant amounts of acid during 24 hr. of heating, and 3 ml. of water were added to each. Control tubes contained resin and water or ovomucoid and water. Acid hydrolysis tubes included for comparison contained ovomucoid and 3 ml. of 0·5N-H_2SO_4 or 2N-HCl. The sealed tubes were constantly mixed at 100° for 0, 0·5, 1, 2, 4, 16, 64 and 128 hr. and the neutral sugars produced separated by filtration or (for acid hydrolysates) by removal of the acid followed by passage through a 900 mg. Dowex-50 column to separate amino sugars and amino acids. Hydrolysis curves for total neutral sugars (anthrone or orcinol methods) showed a maximum at 4 hr. (H_2SO_4), a plateau from 4 to 64 hr. (resin) and progressive destruction (HCl). Maximum orcinol colour values for hexoses were similar for the H_2SO_4 and resin methods but the yield of free hexoses following paper chromatography and elution (Wyatt, 1951) was greatest for the resin method. Hydrolysis of neutral and amino sugars proceeded at the same rate. With ovomucoid, the yield of hexosamine after resin hydrolysis was 95% of that given by HCl hydrolysis and was partly in the form of *N*-acetylglucosamine. No galactosamine was found by a sensitive method (Stoffin & Jeanloz, 1954). Identified sugars recovered were mannose 3·3%, galactose 0·9%, D-glucosamine 9·2%. The resin method is convenient for analysis of small amounts of mucoproteins and has since been applied to purified mucoproteins from normal human plasma (Schmid, 1953*a*, *b*).

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Metabolic Observations on the Erythrocytes from Cases of Galactosaemia. By V. SCHWARZ, L. GOLBERG, G. M. KOMROWER and A. HOLZEL. (*Departments of Pathology and Child Health, University of Manchester*)

Galactosaemia is a syndrome which results from a specific inability to metabolize galactose normally. By studying the behaviour of erythrocytes from cases manifesting this abnormality an attempt has been made to define the nature of any block which may be present in the normal pathway of galactose metabolism.

Red cells from normal subjects, suspended in Krebs–Ringer buffer containing sub-optimal amounts of glucose (9·4 mg./100 ml.) with and without added galactose show a slight but definite increment of respiration due to galactose, amounting to 4·0–12·2 μl./hr./10^{10} cells. In three experiments with cells from galactosaemic children there was a slight inhibition of glucose respiration when galactose was present. These preliminary observations encouraged the hope that the erythrocyte evinces, to some degree at any rate, the bmetaolic aberrations of the liver and possibly other body cells in galactosaemia.

A comparison was made of hexose phosphates in barium–ethanol precipitates of deproteinized haemolysed red cells from normal individuals and from cases of galactosaemia before and after milk or galactose. In adults and normal children on a milk-containing diet the concentration of glucose 1-phosphate (G 1-P) was about 1 mg. and of galactose 1-phosphate (Ga 1-P) 0–0·5 mg./100 ml. blood. In the cells of galactosaemic children before milk or galactose the concentrations of the two phosphates were 1–1·2 and 0–1·8 mg./100 ml. respectively. On a milk or galactose-containing diet, the concentrations were 1–2 and 9–20 mg./100 ml. respectively. Some accumulation of Ga 1-P by galactosaemic cells was also observed *in vitro* after incubating them with galactose. On the other hand the level of G 1-P was not raised.

These results suggest that it is the failure to transform Ga 1-P to G 1-P which constitutes the metabolic block in galactosaemia; and that this may account for some of the clinical and pathological features of the condition.

This work was supported by a grant from the Cow & Gate fund.

The Enzymic Decarboxylation and Interconversion of the Isomers of αα′-Diaminopimelic Acid. By D. S. HOARE. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

α, α′-Diaminopimelic acid (DAP) isolated from *Corynebacterium diphtheriae* was found to be optically inactive (Work, 1951). Following the resolution of synthetic DAP into its three constituent isomers (Work, Birnbaum, Winnitz & Greenstein, 1955), it was established that this naturally occurring DAP was the *meso* isomer. *Meso*-DAP is decarboxylated to L-lysine by a constitutive enzyme from coliform bacteria (Dewey & Work, 1952; Dewey, Hoare & Work, 1954).

A partially purified DAP decarboxylase preparation from *Aerobacter aerogenes* was found to be relatively specific for the *meso* isomer. No significant decarboxylation of the DD or LL isomers was observed under the normal assay conditions, although with very high enzyme concentrations the LL isomer was decarboxylated.

In contrast, Dr E. Work had found (private communication) that the enzyme in acetone-dried cells of *A. aerogenes* appeared to decarboxylate not only the *meso* but also the LL isomer; using the manometric technique, an initial 'lag period' was observed. This has now been confirmed with a number of enzyme preparations, although an initial lag period was not always observed. Furthermore, examination of the reaction products by paper chromatography established that LL-DAP and *meso*-DAP were converted into cadaverine (the latter arising from the action of lysine decarboxylase which is also present).

The 'apparent decarboxylation' of LL-DAP by acetone-dried cells of *A. aerogenes* was not due to the non-specificity of the L-lysine decarboxylase. The L-lysine decarboxylase of *Bacillus cadaveris* and a purified preparation from *Escherichia coli*, both free from *meso*-DAP decarboxylase, did not attack LL-DAP. Further, acetone-dried cells of *Sarcina lutea*, which lack lysine decarboxylase, converted LL-DAP into lysine.

In all the enzyme preparations which have been found to decarboxylate LL-DAP, the rate at which it was decarboxylated was always less than that of the *meso* or natural isomer. Use was made of a lysine requiring mutant *Esch. coli* 26/26 which lacks DAP decarboxylase (Davis, 1952; Dewey & Work, 1952) to demonstrate that the initial stage in the 'apparent decarboxylation' of LL-DAP was its conversion to the *meso* isomer. Acetone-dried cells of *Esch. coli* 26/26 did not decarboxylate LL-DAP. A mixture of these cells and the partially purified *meso*-DAP decarboxylase converted LL-DAP to lysine. DD-DAP was not metabolized.

The sequence of reactions involved in the conversion of LL-DAP into cadaverine by acetone-dried *A. aerogenes* is:

(1) Interconversion: LL-DAP → *Meso*-DAP.
(2) Decarboxylation: *Meso*-DAP → L-lysine.
(3) Decarboxylation: L-lysine → Cadaverine.

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Some Effects of 2:4-Dinitrophenol on the Carbohydrate Metabolism of Tobacco Leaf Disks.

By V. C. RUNECKLES and H. K. PORTER. (*Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S.W. 7*)

Respiration of tobacco leaf disks, floated on water in Warburg respirometers in an atmosphere of oxygen at 25°, was at first stimulated and then inhibited by increasing concentrations of 2:4-dinitrophenol (DNP), at pH 4·6. The increase was about 20% up to 5×10^{-6}M-DNP and about 90% at 5×10^{-5}M. Starch was, at first, the principal respiratory substrate but above 8×10^{-6}M-DNP, starch loss was strongly inhibited. There was a small replacement of starch by sugar, but total carbohydrate loss became insufficient to account for all the CO_2 evolved over the range of maximum stimulation of respiration.

Disks cut from plants depleted of carbohydrate and then supplied with 5% sucrose showed a response to DNP qualitatively similar to that of normal leaves. In both cases R.Q.'s were a little above 1·0 and fell as respiration was stimulated by DNP. About 25% of the sugar taken up into starved leaf disks was converted into products as yet unidentified; simultaneously sugar accumulated to concentrations much higher than those normally occurring and starch was synthesized. Above 10^{-5}M-DNP all three processes were strongly inhibited so that the total amount of sugar utilized was falling over the range of maximum stimulation of respiration.

In the absence of added sucrose respiration was about half that in its presence and the R.Q. was about 0·7. Response to DNP was scarcely affected by omitting sucrose and there was a large stimulation of respiration between DNP concentrations of 5×10^{-5} and 5×10^{-4}. Taken together the data suggest that oxidation of starch or sucrose was gradually inhibited as DNP concentration was increased and that carbohydrate was progressively replaced as substrate by non-carbohydrate material. This material appeared to be the major source of the CO_2 output induced by DNP. This conclusion is supported by the observations that, as DNP concentration increased, R.Q.'s, in the presence of carbohydrate substrate fell, and that the specific activity of the CO_2 evolved, when radioactive sucrose was supplied, also fell, indicating increasing utilization of unlabelled material.

In yeast (Simon, 1953) and in plant tissues (Beevers, 1953) R.Q.'s were found to increase as respiration was stimulated by dinitrophenols, observations which led these authors to postulate increased glycolysis and production of aerobic fermentation. In tobacco leaf no evidence was found of increased glycolysis, on the contrary sugar uptake and carbohydrate utilization were inhibited.

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The Association of Various Soluble Enzymes with Particles in Bacterial Extracts. By T. J. BOWEN and S. DAGLEY. (*Department of Biochemistry, University of Leeds*)

Bowen & Dagley (1954) examined, in the analytical ultracentrifuge, extracts of *Aerobacter aerogenes* harvested from a citrate + mineral salt medium and showed that citridesmolase activity was associated with particles sedimenting at a similar rate to that of a 20 *S* peak previously reported (Siegel, Singer & Wildman, 1952; Billen & Volkin, 1954). Careful examination has now linked activity with a peak not identical with 20 *S* but sedimenting between the well-defined 8 *S* and 29 *S* peaks (Schachman, Pardee & Stanier, 1952); it has an uncorrected sedimentation constant of approximately 12 *S*. The size of the peak depends upon conditions of growth of the bacteria from which extracts were prepared. When the medium used contained glucose + peptone the peak was well defined; it was much smaller for glucose + mineral salt media; and when the cells grew on peptone alone it was not observed.

We have located other enzymic activities using methods previously described (Bowen & Dagley, 1954); briefly, by showing that (*a*) the supernatant became inactive when the '12 *S*' peak sedimented to the bottom of the cell, and (*b*) the activity could be recovered from the deposit. The following enzymes, developed in media as stated, sedimented with the '12 *S*' peak: (1) oxaloacetic decarboxylase of *A. aerogenes*; citrate + mineral salts: (2) glutamic and arginine decarboxylases of *Escherichia coli*; glucose + peptone: (3) citridesmolase of *Esch. coli*; glucose + peptone + citrate.

It is evident that either a number of adaptive enzymes are located on one type of particle, or that they sediment as individuals at about the same rate. Present evidence does not support the latter view since precipitation of extracts with ammonium sulphate, followed by solution in phosphate buffer, altered considerably the distribution of activities amongst sedimenting fractions and appeared to increase greatly the proportion of low molecular weight material.

It is a pleasure to acknowledge the assistance of Mr J. Sykes.

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The Non-Catalysed Hydrolysis of Phosphate Esters. By P. A. T. SWOBODA* and E. M. CROOK. (*Department of Biochemistry, University College, London*)

Primary alkyl esters of orthophosphoric acid although stable to heating under alkaline conditions, may be hydrolysed at 100° in acid. Malengreau & Prigent (1911) first demonstrated for glycerophosphate that this hydrolysis is faster in weak than in strong acids. The rate of hydrolysis, which is negligible at pH 8, rises to a broad maximum at pH 3–4, is a minimum at pH 0·5 and increases again in more acid solutions (Bailly, 1942). The maximum is attributed to the decomposition of the singly ionized species of glycerophosphate, $(C_3H_5(OH)_2.OPO_3H)^-$, which predominates at this pH.

We have investigated the kinetics of this reaction for three esters of biochemical interest: glycerol 1-phosphate, glycerol 2-phosphate and ethanolamine phosphate. The hydrolysis of these esters has been followed both by the colorimetric estimation of inorganic phosphate and by the determination of glycerol or ethanolamine using the method of periodate oxidation.

* In receipt of a grant from the Medical Research Council.

Analysis of the hydrolysate of glycerol 2-phosphate showed the formation of glycerol, identified by isolation, and orthophosphate, demonstrated by potentiometric titration with the glass electrode. No evidence was found for the presence of monometaphosphate in appreciable amount, although the possibility remains that it is a transient intermediate in the reaction. A quantitative first order dependence of rate on the concentration of the singly ionized species of glycerol 2-phosphate has been demonstrated in the pH range 3–7. The reaction was not acid catalysed and the rate independent of ionic strength. No base catalysis was observed either by negatively charged acetate ions or neutral aniline molecules. Similar results were obtained with the other esters. Rate constants were determined at ten degree intervals from 80° to 110°. The calculated values at 100° were $1{\cdot}35 \times 10^{-5}$, $2{\cdot}77 \times 10^{-5}$ and $3{\cdot}07 \times 10^{-5}$ sec.$^{-1}$ for the singly ionized species of glycerol 1-phosphate, glycerol 2-phosphate and the zwitterion of ethanolamine phosphate respectively. Activation energies of 29·9, 30·3 and 29·6 kcal. mole^{-1} were found, the

Arrhenius frequency factor having values of $4{\cdot}28 \times 10^{12}$, $15{\cdot}08 \times 10^{12}$ and $6{\cdot}71 \times 10^{12}$.

In collaboration with the Chemistry Department of University College London, the hydrolysis was studied in water enriched in ^{18}O. Provisional results indicate cleavage of the ester between the phosphorus and oxygen atoms (Bunton, C. A., Crook, E. M., Lewis, T. A., Llewellyn, D. R., Swoboda, P. A. T. & Vernon, C. A., unpublished). This is not at variance with the experimentally observed insensitivity of the reaction to changes in structure of the alkyl radical.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **337th Meeting** *of the Biochemical Society was held at the London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1, on Saturday, 19 February 1955, at 11 a.m. and took the form of a Symposium on 'The Biochemistry of Vitamin B_{12}'.*

COMMUNICATIONS

Isolation and Chemistry of Vitamin B_{12}. By E. LESTER SMITH. (*Glaxo Laboratories Ltd, Greenford, Middlesex*)

The isolation of vitamin B_{12} from liver extracts and fermentation liquors will be described briefly. Vitamin B_{12} contains a —CN group directly linked to cobalt; its replacement by other acid groups or by —OH gives rise to a series of 'cobalamins'. The presence of 5:6-dimethyl-1-(α-D-ribofuranosyl)benziminazole-2′(or -3′)-phosphate has been established by degradation and synthesis. This 'nucleotide' fragment can be removed intact by brief treatment with warm concentrated hydrochloric or perchloric acids, leaving a coloured substance proved identical with factor B, which has been previously extracted from faeces and from a fermentation source.

Either vitamin B_{12} or this factor B can be progressively hydrolysed by cold dilute mineral acid or alkali with elimination of ammonia from four $CONH_2$ groups, leaving two series of acids that can be fractionated by electrophoresis on paper and partition chromatography. Eight acids (three isomeric mono-, three di-, one tri- and one tetrabasic) in the series retaining the nucleotide have been crystallized. Several of these have been reconverted into vitamin B_{12} by ammonolysis of their ethoxyformic anhydrides.

Continued hydrolysis releases DG-1-amino-2-propanol. Drastic alkaline hydrolysis gives a product with six carboxyl groups; this has been crystallized by Sir Alexander Todd's group at Cambridge.

The 'chromophore' can be altered in ways not yet elucidated, by treatment with halogens or hot alkali. The cobalt is firmly bound, and its removal (e.g. by ascorbic acid or by strong oxidizing agents) appears to disrupt the molecule.

The 2- to 5-carbon acids recognized among the permanganate oxidation products provide little clue to structure; X-ray crystallography has revealed something resembling a porphyrin, but with one methene carbon missing from the macro-ring.

Other Factors Related to Vitamin B_{12}. By S. K. KON. (*The National Institute for Research in Dairying, The University of Reading*)

Micro-organisms, ultimately responsible for the vitamin B_{12} found in animal tissues, also produce compounds related to it but differing in the nature of the nucleotide base of the molecule. These compounds occur naturally in rumen and gut contents, sewage sludge and other products of microbial fermentation. Several have been isolated and characterized, many others await identification. Those of known constitution contain a purine in the nucleotide instead of the dimethylbenziminazole of vitamin B_{12} itself. The purine and benziminazole families are linked through a parent substance, factor B, that has no nucleotide. The natural vitamin B_{12}-like compounds so far isolated in the pure state are inactive for higher animals, with the exception of vitamin $B_{12\,III}$ from sewage sludge, but are active to a different extent for different micro-organisms; their resolution and separation rests on differential microbiological assay and on chromatographic and electrophoretic procedures. Given suitable precursors certain organisms, among them some derived from the gut, are capable of synthesizing not only the 'natural' B_{12}-like compounds but also 'unnatural' ones containing derivatives of benziminazole or purine. Some of the benziminazole series are active for higher animals and in pernicious anaemia. Some of the unnatural ones may well have antivitamin B_{12} properties. It is likely that the 'natural' compounds play the part of vitamin B_{12} in bacterial metabolism, but their function has not yet been established. Their virtual absence from animal tissues indicates selective mechanisms of uptake probably linked with the formation of complexes akin to that of vitamin B_{12} with the intrinsic factor. Complexes of such nature probably exist within microbial cells.

The Measurement of Vitamin B_{12}. By M. E. COATES and J. E. FORD. (*The National Institute for Research in Dairying, The University of Reading*)

The discovery in natural materials of a large and growing family of vitamin B_{12}-like compounds, having widely different activities towards different test reagents, chemical, biological and microbiological, complicates the problems of assay. Higher animals are narrowly selective in their requirement for 'vitamin B_{12}'; and since generally the results of the tests will be interpreted in terms of the requirements of man or beast, it is essential to be aware of the limitations of the assay methods employed.

Tests with higher animals are not necessarily inherently specific, and results are likely to be complicated by the intrusion of unidentified growth factors. But in suitable conditions they have a special value in that they measure the availability of the vitamin in its naturally occurring form, and do not involve the drastic extraction procedures necessary for the chemical and microbiological assays. On the other hand, they are expensive and time-consuming, and not suited to routine application except as a necessary check on the microbiological and chemical methods.

Most of the latter are highly susceptible to interference from the vitamin B_{12}-like compounds, and in some instances from other non-specific substances. In some applications the use of non-specific techniques can be justified by the known absence of interfering substances. But for general use the test must discriminate against substances inactive for higher animals.

Micro-organisms are known that have an animal-like requirement for 'vitamin B_{12}'. The protozoan *Ochromonas malhamensis* is one such, and it has been recommended as a test organism.

The different assay methods will be discussed, with special reference to the problem of specificity of test. Certain similarities in the mechanisms underlying the selective absorption of 'vitamin B_{12}' in living organisms will be considered.

The Absorption and Excretion of Vitamin B_{12}. By D. L. MOLLIN and S. J. BAKER. (*Department of Haematology, Postgraduate Medical School of London*)

The fact of B_{12} absorption can be demonstrated by following the haematological responses and changes in the serum B_{12} concentration of patients with B_{12} deficiency after oral doses of the vitamin, but the absorption of physiological amounts of B_{12} can only be measured by balance studies with radioactive B_{12} (Heinle, Welch, Scharf, Meacham & Prusoff, 1952; Callender, Turnbull & Wakisaka, 1954; Schilling, 1954). Repeated studies in the same individual are possible if B_{12} is labelled with shorter-lived isotope of cobalt (^{58}Co) (Bradley, Lester Smith, Baker & Mollin, 1954). Using this material it can be shown that most normal subjects absorb 0·3 μg. or more from a test dose of 0·6 μg. Patients with pernicious anaemia absorb less than 0·12 μg. unless the material is given with a source of intrinsic factor. 'Carbachol' (carbamylcholine chloride), which increases the production of intrinsic factor in normal subjects, does not do so in patients with pernicious anaemia. The amount of B_{12} that is absorbed by a given amount of intrinsic factor varies in different patients, but in any one patient the amount absorbed is directly proportional, over a certain range, to the amount of intrinsic factor given. The implication of this for the assay of intrinsic factor will be discussed.

Schilling (1953) introduced an alternative method for demonstrating the absorption of radioactive B_{12}. The use of this method for measuring the amount of B_{12} absorbed will also be discussed.

The amount of 'B_{12}' excreted in the urine of normal subjects and of patients with B_{12} deficiency after parenteral injections of non-radioactive and radioactive B_{12} varied, but no significant difference was found in the amounts excreted by each group.

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The Intrinsic Factor. By A. L. LATNER. (*Department of Pathology, King's College, University of Durham*)

After a short historical introduction, reference will be made to available methods of assay; by clinical observation and those involving the use of radioactive vitamin B_{12} (Heinle, Welch, Scharf, Meacham & Prusoff, 1952; Schilling, 1953; Glass, Boyd, Gellin & Stephanson, 1954). Work relating to the site of production of intrinsic factor will be discussed, as well as methods concerned with its

isolation. The most potent product so far described is mucoprotein in nature (Latner, Merrills & Raine, 1954). It has blood-group substance activity, and acts as a growth factor for *L. bifidus*. It is homogeneous in the ultracentrifuge and after paper-strip electrophoresis at different pH levels, and has been further purified by precipitation with alcohol at low temperatures. An account will be given of its chemical composition and properties.

In conclusion, the possible mode of action of intrinsic factor will be discussed, from the points of view of theories relating to: (*a*) an interaction product, (*b*) vitamin B_{12} binding, (*c*) activated vitamin B_{12}, and (*d*) catalysed absorption (Castle, 1953). In this respect, an account will be given of recent investigations with gastrectomized rats.

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The Function of Vitamin B_{12} in Animal Metabolism. By H. R. V. Arnstein. (*National Institute for Medical Research, London, N.W.* 7)

Although vitamin B_{12} influences the metabolism of carbohydrate, fat, protein and nucleic acids in animals, few details are known about the fundamental mechanisms of these effects.

It is now well established that the biosynthesis of labile methyl groups by rats and chicks is facilitated by vitamin B_{12}, but transmethylation is probably not affected (Smith, 1954). This reaction satisfactorily explains the improved utilization of proteins deficient in methionine and the reduced choline requirement in the presence of vitamin B_{12}.

Vitamin B_{12} must, however, have additional functions, since it is not completely replaced by methionine. A more fundamental function appears to be the reduction of disulphide groups, as shown by the decreased levels of soluble sulphydryl compounds, particularly glutathione, in vitamin B_{12} deficiency (Ling & Chow, 1953; Register, 1953). The stimulation of methionine synthesis may be due, at least in part, to increased levels of homocysteine, which is a likely acceptor of the methyl group precursor.

Although the biosynthesis of labile methyl groups is probably normally unimportant, closely related reactions of a similar type may be of greater significance. The methyl group of thymine is synthesized from serine (Elwyn & Sprinson, 1954), which is quantitatively the most important source of newly synthesized methionine methyl groups (Arnstein & Neuberger, 1953). The biosynthesis of both these methyl groups involves the transfer of formaldehyde and the reduction of a hydroxymethyl group. Pteroylglutamic acid functions in the transfer of formaldehyde and it seems possible that vitamin B_{12} may be concerned in the subsequent reduction.

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The Function of Vitamin B_{12} in Micro-organisms. By June Lascelles and M. J. Cross. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The ability of compounds of different chemical type to replace B-group vitamins for growth of micro-organisms gives evidence that they are products of reactions in which the growth factor in question is concerned (Woods, 1952). Experiments of this kind suggest that vitamin B_{12} is required for the synthesis of methionine and nucleic acid components, particularly deoxyribosides. Organisms investigated fall into two groups—those whose B_{12} requirement is satisfied by methionine and which do not respond to deoxyribosides, and those for which only deoxyribosides are active. The latter need methionine even in the presence of the vitamin.

Methionine but not homocysteine completely replaces B_{12} for growth of mutant strains of *Escherichia coli*. Evidence that the vitamin is concerned at the methylation stage comes from experiments with deficient cell suspensions of this

organism, which transform homocysteine to methionine only in the presence of B_{12}. Thymidine or other deoxyribosides replaces the vitamin for growth of some lactobacilli (e.g. *Lactobacillus leichmannii*); these organisms contain transdeoxyribosidases even when grown without B_{12}. Some of them do not grow on thymidine unless purines are also present.

The function of vitamin B_{12} seems to be closely connected with that of the folic acid group of factors; this is emphasized by the effect of B_{12} in reducing the amount of *p*-aminobenzoic acid required to promote growth and overcome sulphonamide inhibition of *Esch. coli*. It is probable, however, that these factors are involved at different loci in biosynthetic reactions.

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FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Nucleotide exchange reactions catalysed by ribonuclease and spleen phosphodiesterase:

1. Synthesis and interconversion of simple esters of ribomononucleotides. By L. A. HEPPEL and P. R. WHITFELD
2. Synthesis of polynucleotides. By L. A. HEPPEL, P. R. WHITFELD and R. MARKHAM

The enzymic hydrolysis of ribonucleoside-2′:3′ phosphates. By P. R. WHITFELD, L. A. HEPPEL and R. MARKHAM

A note on the structure of triphosphopyridine nucleotide. By L. A. HEPPEL, P. R. WHITFELD and R. MARKHAM

Provitamin D in animal tissues. By G. N. FESTENSTEIN and R. A. MORTON

Cholesta-3:5-dien-7-one in human atherosclerotic aortas. By N. L. KANTIENGAR and R. A. MORTON

Formation of cholesta-3:5-dien-7-one in rats. By N. L. KANTIENGAR and R. A. MORTON

The administration to rats of cholesta-3:5-dien-7-one. By N. L. KANTIENGAR and R. A. MORTON

The effects of administering cholesterol and cholesta-3:5-dien-7-one to cockerels. By N. L. KANTIENGAR, J. S. LOWE, R. A. MORTON and G. A. J. PITT

The partial purification of leaf ribonuclease. By MARGARET HOLDEN and N. W. PIRIE

The preparation of ribonucleic acid from yeast, tobacco leaves and tobacco mosaic virus. By MARGARET HOLDEN and N. W. PIRIE

A comparison of leaf and pancreatic ribonuclease. By MARGARET HOLDEN and N. W. PIRIE

Metabolism of polycyclic compounds. 9. Metabolism of 2-naphthylamine in rat tissue slices. By J. BOOTH, E. BOYLAND and D. MANSON

The oxidation of glutathione by a lipoxidase enzyme from pea seeds. By L. W. MAPSON and E. M. MOUSTAFA

The free amino acids of fish. 1-Methylhistidine and β-alanine liberation by skeletal muscle anserinase of codling (*Gadus callarias*). By N. R. JONES

The metabolism of citric acid in the mammary gland:

1. The effect of *p*-nitrophenol on the synthesis and oxidation of citric acid by homogenates of the mammary gland. By C. TERNER
2. The effect of *p*-nitrophenol and of fluoride on the synthesis of citric acid in fluoroacetate-blocked homogenates. By C. TERNER

Effects of adrenal corticoids on fatty acid synthesis in mammary gland slices *in vitro*. By MARY L. MCNAUGHT, R. F. GLASCOCK, JUDITH H. BALMAIN and S. J. FOLLEY

Cacao polyphenolic substances. 3. Separation and estimation on paper chromatograms. By W. G. C. FORSYTH

The metabolism of sulphated polysaccharides in limb atrophy in the rat. By H. G. B. SLACK

The use of radioactive isotopes in immunological investigations. 7. Labelled proteins and protein antigens containing both iodine and mustard gas sulphone groups. By G. E. FRANCIS, W. MULLIGAN and A. WORMALL

The uncoupling of oxidative phosphorylation in rat-liver mitochondria by thyroxine, triiodothyronine and related substances. By H. G. KLEMPERER

The uptake of thyroxine and triiodothyronine by rat-liver mitochondria. By H. G. KLEMPERER

The mechanism of itaconic acid formation by *Aspergillus terreus*:

1. The effect of acidity. By H. LARSEN and K. E. EIMHJELLEN
2. The effect of substrates and inhibitors. By K. E. EIMHJELLEN and H. LARSEN

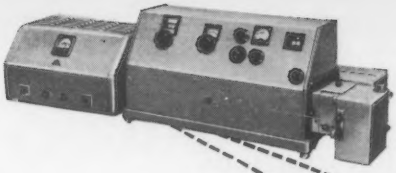
Biosynthesis of fatty acids in cell-free preparations:

2. Synthesis of fatty acids from acetate by a soluble enzyme system prepared from rat mammary gland. By G. POPJÁK and ALISA TIETZ
3. Coenzyme A dependent reactions in a soluble enzyme system of mammary gland. By ALISA TIETZ and G. POPJÁK

Studies on the use of sulphonated cation exchange resins for the hydrolysis of ovomucoid. By A. ST J. DIXON

The reduction of esters of simple peptides by metal hydrides. By J. LEGGETT BAILEY

Systematic degradation of peptides from the carboxyl end. By J. LEGGETT BAILEY



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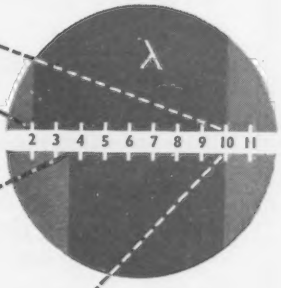

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Book List

Biochemistry of the Aminosugars

By P. W. Kent, M.A., B.Sc., Ph.D., D.Phil., F.R.I.C., and M. W. Whitehouse, B.A., B.Sc., A.R.I.C., Dept. of Biochemistry, Oxford.

The book surveys the biochemistry of mucins and other aminosugar-containing substances and includes a consideration of their metabolism and of the enzymes which act on them and their derivatives. The first part of the book discusses aminosugars and muco-substances in the biological environment, while the second part treats the chemistry (detection, estimation, structure and reactions) of aminosugars. The work is not solely confined to glucosamine and galactosamine and an attempt has been made to collate the properties of all known aminosugars, both natural and synthetic. *Price* 40*s.*

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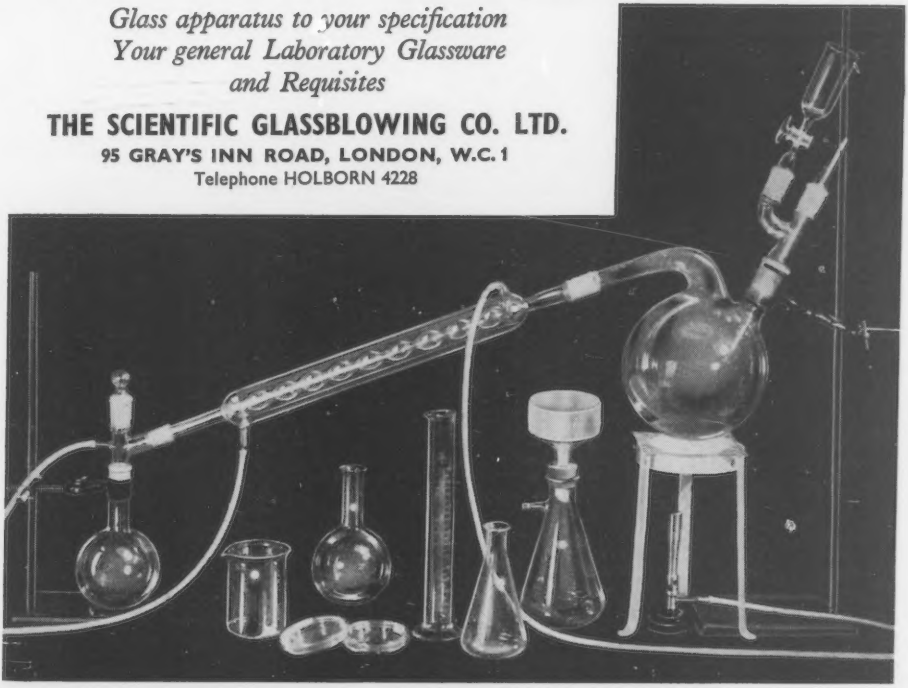

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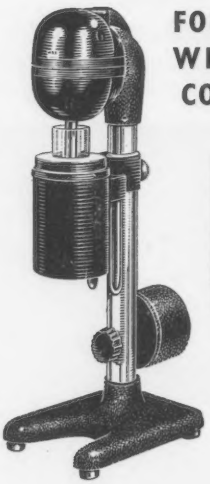

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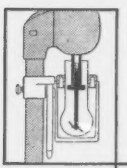

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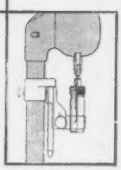

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